Oral Presentation (JSPS-6)

Influenza D virus and bovine coronavirus play important roles in bovine respiratory disease in Japan

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Keywords: antigenicity, bovine coronavirus, BRD, influenza D virus, viral metagenomics.

INTRODUCTION

Bovine respiratory disease (BRD) is one of the most commonly diagnosed causes of morbidity and mortality in the cattle industry. Co-infection with several viruses and bacteria causes BRD. Despite the use of antibiotics and several commercial vaccines against viruses, such as bovine respiratory syncytial virus (BRSV), bovine viral diarrhea virus (BVDV) 1 and 2, bovine herpesvirus 1, bovine adenovirus 7 and bovine parainfluenza virus 3, BRD remains the most common and costly problem in Japan. Therefore, we suspect that viruses not treated by the vaccines are a possible cause of BRD.

Metagenomic analysis has recently allowed comprehensive viral characterization of the bovine respiratory tract. Ng et al. reported that bovine adenovirus 3, bovine rhinitis A virus and influenza D (FluD) virus were significantly associated with BRD in the USA [1]. Mitra et al. reported that FluD virus was significantly associated with respiratory disease, and viruses that are commonly associated with BRD, such as BVDV, bovine herpesvirus 1, BRSV, and bovine parainfluenza virus were detected less frequently in Mexico and the USA [2]. Although the viral pathologies and clinical diseases in sole infection appear mild, these results suggest that they play an essential role as a trigger of BRD.

To identify which viruses were associated with BRD in Japan, we first performed a viral metagenomic analysis using nasal swab samples from respiratory-diseased cattle. The results suggested that bovine coronavirus (BCoV) and FluD virus played a significant role. Therefore, we conducted a molecular-epidemiological survey of nasal swab samples from respiratory-diseased and healthy cattle to assess the contribution of BCoV and FluD virus. Finally, we performed a phylogenetic analysis and assessed the antigenicity.

MATERIALS AND METHODS

Nasal swab samples were collected from 42 farms in the Aichi, Mie, Kagoshima, Kumamoto and Miyazaki prefectures in Japan from 2016 to 2018. All of the farms had at least one or more respiratory-diseased subjects.

A total of 46 samples from 23 farms with respiratory-diseased cattle were used for the viral metagenomic analysis. After removing the host-derived nucleic acids and performing RNA extraction, cDNA was synthesized by a random hexanucleotide primer. After purification, amplification and quantification, dsDNA was subjected to library preparation. Then, next-generation sequencing was performed using a MiSeq instrument (Illumina). The sequence generated by MiSeq was analyzed using the CLC Genomics Workbench 9 software (Qiagen).

After RNA extraction and reverse transcription from nasal swab samples, qPCR was performed to detect and quantify the BCoV and FluD viral genes. Probe qPCR Mix (TaKaRa Bio) and PrimeTime qPCR Assays (Integrated DNA Technologies) were used to detect the BCoV, FluD virus and bovine □-actin genes. The full-genome sequences of the FluD virus were determined by the next-generation sequencing method. The partial sequence (411bp) of the polymorphic region of BCoV spike (S) gene was determined by the capillary sequence method. The viral genome sequence that was determined in our study and the available genome sequences that we retrieved from GenBank were aligned using ClustalW. Molecular phylogenetic trees were constructed using the neighbor-joining method with MEGA7 software.

A total of 5 BCoV were isolated from the nasal swab samples using HRT-18G cell. Antisera against the reference BCoV strain (strain 66) were made form naive cattle. A hemagglutination (HA)-inhibition (HI) test was performed to compare the antigenicity of epidemic and reference bovine coronavirus.

Statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software). p <0.05 was considered statistically significant in this study.

RESULT AND DISCUSSION

The greatest number of detected viral genomes were as follows: bovine rhinitis B virus > BCoV > bovine parainfluenza 3 > BRSV > bovine rhinitis A virus > bovine adenovirus 3 > Flu D virus. Bovine parainfluenza 3 and BRSV are included in the commercial respiratory vaccines in Japan. Therefore, we focused on BCoV and FluD viruses in this study. However, we are planning to analyze the bovine rhinitis virus and bovine adenovirus 3 in the near future.

We found that 2.1% (8/377) and 18.6% (85/455) of cattle were infected with FluD virus and BCoV, respectively. Fifty-eight of the 273 (21.2%) cattle with respiratory symptoms were PCR positive for BCoV, with a medium value of viral copies of 206.0 (Av.=5,691.7 copies). On the other hand, 27 of the 182 (14.8%) healthy cattle were also PCR positive, with a medium value of viral copies of 43.6 (Av.=886.3 copies). The viral copies of cattle with respiratory symptoms were higher than those of healthy cattle (p=0.06, Wilcoxon signed-rank test). Three of the 172 (1.7%) cattle with respiratory symptoms were positive for FluD virus, with Cq values of 29, 33 and 36 (Av.=32.8). However, 5 of the 205 (2.4%) healthy cattle were also positive for this virus, with Cq values ranging from 31 to 35 (Av.=34.4). Although the mean Cq values of cattle with respiratory symptoms were lower (viral loads were higher) than those of healthy cattle, there were no significant differences (p=0.42, Student's t-test).

Full-genome sequence analysis revealed that the FluD virus that was isolated in Japan formed an individual cluster that was distinct from strains found in other countries [3]. This result suggests that this virus may have evolved uniquely in Japan over an extended period of time and that the viral pathology of the Japanese strains may be different from that of strains found in other countries. Based on the partial sequence of the BCoV S gene, all of the epidemic viruses were clustered into genotype 4 with the Japanese BCoV strain in 2004-2008 and different from available the BCoV diarrhea vaccine strain in genotype 1. The epidemic viruses were divided into two subgroups in genotype 4 (named genotype 4-1 and 4-2) and separated from the Japanese strain in 2004-2008. The antigenicity of FluD virus in Japan has already been reported by Horimoto's group [4]. Therefore, we analyzed the antigenicity of BCoV. We successfully isolated 5 BCoV that belong to genotype 4-1 or 4-2. Antisera against the reference BCoV (genotype 1) were made from naive cattle. Now, hemagglutination (HA)-inhibition (HI) tests are being performed to compare the antigenicity of the epidemic and reference bovine coronavirus.

CONCLUSION

To prevent losses that result from BRD, it is vital to monitor pathogens that are associated with BRD. From our results, BCoV might be associated with BRD in Japan, and its antigenicity was gradually separated from the past epidemic strains. We also found that the FluD virus was epidemic in Japan and might be related to a part of BRD. However, the viral antigenicity of Japanese strains might be different from that of strains found in other countries. Continuous surveillance is required to determine the importance of these viruses.

ACKNOWLEDGMENTS

This work was supported by JSPS Core-to-core Program, B. Asia-Africa Science Platform.

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